Organ Fabrication Using Pigs as An in Vivo Bioreactor

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We present the most recent research results on the creation of pigs that can accept human cells. Pigs in which grafted human cells can flourish are essential for studies of the production of human organs in the pig and for verification of the efficacy of cells and tissues of human origin for use in regenerative therapy. First, against the background of a worldwide shortage of donor organs, the need for future medical technology to produce human organs for transplantation is discussed. We then describe proof-of-concept studies in small animals used to produce human organs. An overview of the history of studies examining the induction of immune tolerance by techniques involving fertilized animal eggs and the injection of human cells into fetuses or neonatal animals is also presented. Finally, current and future prospects for producing pigs that can accept human cells and tissues for experimental purposes are discussed. (DOI: 10.2302/kjm.2019-0006-OA; Keio J Med 69 (2) : 30–36, June 2020)

Keywords: organ fabrication, donor shortage, in vivo bioreactor, pig, stem cell

Introduction

Organ transplantation has become well established as “the wonder treatment” of the 20th century as a final treatment for patients with organ failure and no other hope for survival.¹ While the prognosis for transplant patients has dramatically improved, there is a chronic worldwide shortage of donor organs. This profound shortage of organs has encouraged the unethical or illegal sourcing of donors. In an early initiative of the 2008 Istanbul Declaration, professionals engaged in organ transplantation demanded the following guarantees: (1) opposition to organ trafficking and transplant commercialism, (2) promotion of transplantation from deceased donors in the same country and/or region, and (3) protection and following up of living donors.² Various efforts have been made in many countries, but 10 years later, the organ shortage has not improved. E.K., one of the authors, has participated in living donor liver transplantation in clinical practice in Japan.³ Living donor transplantation was developed as the sole alternative in the absence of a deceased do-

nor and has saved many lives. However, there is a great demand for next-generation medical treatments to render organ transplantation unnecessary, e.g., promotion of the concept of the production of “transplantable organs” in patients themselves and research and development of specific techniques to this end.⁴

Much progress has been made in recent years in the fundamental research of stem cells. Techniques for the test-tube production of organs from pluripotent cells can be applied to humans. Human embryonic stem cells (ESCs)⁵ and human induced pluripotent stem cells (iPSCs)⁶ have been produced as human pluripotent stem cells. These achievements are promising for research into the production of vital organs in the laboratory. One current issue is the need for reactors that provide the infrastructure for organ development and also supply oxygen and nutrition for the continued production and growth of organs. The pig has been identified as the animal most likely to provide the required in vivo reactor infrastructure. As the next step, pigs that can accept human cells and tissues must be produced. To achieve this, techniques for suppressing im-

munity in the pig to enable acceptance of human cells and for inducing immune tolerance to cells must be evaluated. Immunosuppression has been successful in pigs^{7–11}; however, unlike for small animals, management of the rearing of these immunocompromised pigs makes organ production scientifically and economically difficult.¹²

In this review, we first provide an overview of recent studies on the production of human tissues and organs in living pigs. After describing studies related to immune tolerance (which involves elimination of immune responses in individual animals), we introduce recent research on the production of pigs that can accept human cells and tissues.

Proof of Concept in Small Animals of Techniques for Growing Human Organs in Pigs

The technology used for producing antibodies or drugs to be used in humans and for producing cells and/or tissues of human origin in the bodies of living animals are referred to as “animal factories” or *in vivo* bioreactors. Pigs have attracted much attention in this respect: in addition to the history and social importance of the hygienic management of the pig as a food animal, in recent years it has also been evaluated for developmental engineering purposes. It is an additional advantage that they give birth to multiple offspring from the economical and ethical points of view.

In studies of transplantation between mice and rats, we successfully replaced, *in vivo*, the livers of mice with rat hepatocytes, which were then transplanted into rats.¹³ Rat hepatocytes were injected into severe combined immunodeficient (SCID) mouse pups. For use as an *in vivo* bioreactor, the mice were subjected to genetic procedures that caused progressive damage to the liver, preventing its development. As the SCID mice grew, the livers were replaced with rat hepatocytes. In the livers of mice produced using this technique, more than 95% of cells were replaced with rat hepatocytes. Although the vascular system and biliary system were still murine, when such livers were transplanted with blood vessels attached into the rat as a recipient, the recipient rat were extremely immunologically viable and showed long-term survival.¹³ However, the mice as *in vivo* reactors must be SCID to accept injected xenogenic rat cells. Evidently, SCID pigs would be required to verify this principle in human-sized pigs. However, the extremely high costs of reproduction and hygiene management of SCID pigs is a considerable limitation to the future development of this approach.

Nakauchi et al. have established a developmental engineering method for heterogeneic mouse–rat transplantation of the pancreas in which the target organ increased¹⁴. In this technique, a key gene associated with organ development in the mouse is knocked out and, after fertilization, the blastocyst of knocked out mice is complemented with rat iPSCs. Mice born using this procedure contain

rat pancreatic islets of Langerhans. Islets of Langerhans taken from the mouse pancreas produced an effect when transplanted into diabetes-induced rats.¹⁴ For this strategy, apancreatic pig that did not develop a pancreas have also been successfully produced, and it has been demonstrated that after blastocyst exchange with a healthy pig, a pancreas is formed from the exogenous cells as the apancreatic pig could not survive for a long time.¹⁵ This technique produces xenogenic chimera at the level of the fetus, and animals capable of development can grow with normal immunocompetence. In recent years, the production of pig–human chimera at the fetal level was also performed at the Salk Institute (USA).¹⁶ They showed that human ESCs or iPSCs could be introduced into fertilized pig eggs to produce a human–pig chimera. However, this technique raises serious ethical questions. Consequently, there are limitations to creating a chimera with a mixture of pig and human cells that are introduced at the fetal level. For human–pig chimeras, it has been said that “such a horrible creature should not be brought to term.” Moreover, the introduction of heterologous cells and genes during insemination must be thoroughly considered, not only from the perspective of legal problems regarding the insemination procedure, but also for the loss of the personal identity of a living being. To better understand the production of the human-pig xenogenic chimera, we summarize these approaches for human cell injection into the pig in **Fig. 1**.

Overcoming the ethical issue of xeno-chimera, the methods for inducing immune tolerance by injecting human cells into pig fetuses have been reconsidered. Numerous studies have examined heterogeneic cell transplantation. Experiments inducing immune tolerance within the same species are free from the ethical and legal problems described above. This history of the concept of immune tolerance is reviewed in the next section.

Historical Background of Immune Tolerance

Medawer, who together with Burnet, won the Nobel Prize in 1960 for Physiology and Medicine, reviewed the progress of immunological tolerance at the time.¹⁷ Medawer first described the work of Owen in 1945. Owen investigated more than 80 pairs of twin calves, nearly all of which had the same blood type,¹⁸ and discovered that individual calves that experience anastomosis in the placenta accepted the exchange of skin grafts after maturity. In multiple fetuses of different sexes, female fetuses, in which there is abnormal differentiation of the reproductive organs causing infertility, are called freemartins and are the result of blood chimerism produced by placental anastomosis. The sex chromosomes show XX/XY chimerism, which is thought to exist at 1–2% in the Holstein breed.

In 1950, Hasek reported that this phenomenon could be reproduced experimentally in different species. He

Fig. 1. Various approaches to human organ development in pigs.

(A) Pigs with human liver created by blastocyst complementation have been introduced as the embryonic approach. Human ESCs or iPSCs can be injected into pig eggs in which the master gene for liver formation has been knocked out. The developing egg is implanted to a pseudopregnant mother pig. The pig fetus has a human liver from the developmental stage because human stem cells engraft the liver site. As a result, pigs with human livers can be produced. Schematics show fetal (B) and neonatal (C) approaches via injection of human cells into fetal or neonatal pig's organ. Immune insufficient pigs with human hepatocytes gradually develop under apoptosis of pig hepatocytes by use of the conditional knockout method. Finally, chimeric human liver is obtained with a pig liver scaffold.

produced parabiosis in fertilized eggs of chicken and Pekin ducks and demonstrated that the birds, which were subsequently hatched, did not produce antibodies when immunized with the blood of the other species.¹⁹ Hasek inferred that, in addition to the blood chimerism discovered by Owen, this phenomenon was induced at this point of the life cycle. In 1953, Medawar et al. conducted experiments in which immune tolerance was established in the fetuses of mice or embryonic chicks by implanting a live antigen.²⁰ They termed this phenomenon “actively acquired tolerance.”

In 1969, at Cambridge University, UK, Calne reported

examples of liver transplantation between pigs with different graft antigens in which tolerance was acquired without the use of immunosuppressants.²¹ At the same time, Calne enabled liver transplantation through the clinical application of immunosuppressants. This phenomenon, named liver tolerance, was advanced by the same group using rats with a clearly defined genetic background.²² Around the same time, Binns, also in Cambridge, evaluated the actively acquired tolerance demonstrated by Medawar in small animals, such as mice, and induced immune tolerance in pig fetuses by implanting them with bone marrow cells or lymphocytes from another pig.²³

He reported that it is difficult to induce immune tolerance unless it is within 60 gestational days. Moreover, the same group attempted to induce an immunosuppressed state by removing the thymus during the neonatal period. In pigs on the contrary, unlike in mice, T cells start to appear during the neonatal period, but it is known that an immunosuppressed state can be induced to a certain extent.²⁴

Mice and pigs differ greatly in body size, leading to large differences in the costs of experimentation. However, previous studies provided a great deal of knowledge on the concept of using pigs as bioreactors and preclinical experimental systems.^{21,25}

Current and Future Prospects for Production of Pigs That Can Accept Human Cells

Pigs are very similar to humans in body size and in the structure and metabolism of the internal organs. Consequently, pigs are extremely important as a preclinical model for verifying the safety and efficacy of products and techniques for regenerative medicine. However, the progress in verifying differentiation induction in human pluripotent cells in recent years has led to the need for transplantation of the resulting cells in immunocompromised animals and pathological verification of tridermic differentiation.²⁶ Currently, multiple immunosuppressants administered in clinical practice to humans (e.g., tacrolimus, mycophenolate mofetil, and steroids) must be used to achieve survival of cells of human origin in pigs.²⁷ However, the immune response is stronger than the human–monkey response,²⁸ and thus severe drug regimens and management of these drugs are necessary.

At Keio University School of Medicine, clinical applications of regenerative therapy using human iPSC-derived cardiomyocytes and neural precursor cells are underway, mainly in the Department of Cardiology and the Department of Orthopedic Surgery, respectively. In this section, we discuss the findings from research on the production of pigs that can accept human cells and tissues, evaluated in the Department of Organ Fabrication at Keio University School of Medicine.

Immunosuppression by total thymectomy in neonatal pigs was first reported in 1972 by Binns et al. The world's smallest experimental miniature pig, the micro-mini pig (MMP), was developed in Japan.^{29–31} Since 2012, total thymectomy has been carried out in MMP piglets delivered by caesarian section in a specific pathogen-free environment, with observation over at least 2 years (**Fig. 2**). MMPs were selected because, even when mature, the animals are smaller than domestic pigs or even ordinary miniature pigs, thereby facilitating long-term monitoring of the safety and efficacy of cells and tissues for regenerative medicine. There was no difference in the body weight during growth between athymic and control MMPs under specific pathogen-free conditions. However, the thy-

mic hormone thymosin- α 1 was maintained at a low level in the athymic MMPs throughout the observation period. In addition, although no significant decrease was noted during flow cytometric verification of T-cell numbers among peripheral lymphocytes, there was a decrease in the *in vitro* proliferation of peripheral lymphocytes in response to pokeweed mitogen. In this model, human hepatocytes were grafted into the athymic MMP liver without using an immunosuppressant, and the effect was verified by measuring peripherally expressed albumin. Whereas, in the control group, human albumin decreased to below the limit of detection within 1 week, human albumin was detected in the peripheral blood of the tested MMPs for 2–3 weeks³². These athymic MMPs are now available commercially, and various laboratories have begun to use them.

We have also carried out surgical improvement in ordinary mature miniature pigs to facilitate grafting of human cells and tissues. In this method, the thymus and spleen of mature miniature pigs are surgically removed and the pigs are fed immunosuppressants through a stomach tube³³ (**Fig. 3**). Artificial blood vessels produced by 3D printing of clumps of human fibroblast cells were surgically inserted and monitored as shunts into the carotid artery and jugular vein of these pigs. Following prior administration of three immunosuppressants, shunt flow in the inserted vessels ceased within 1 month in all animals with intact thymus and spleen (control group), but for the system described above, favorable blood flow was maintained for several months in operatively immunodeficient pigs. We refer to these pigs as operational SCID pigs, because they are produced by using surgical procedures and immunosuppressant administration.³³

Methods for inducing immune tolerance by injecting a human antigen into pig fetuses or neonatal piglets are also being developed. The initial point of reference for the method of injecting human cells into the thymus is a historic experiment in small animals carried out by Posselt et al. in 1990. After causing the collapse of the immune system by extreme administration of antilymphocyte serum, target donor islets were injected into the thymus (because the thymus has an immunoisolating capacity) and immune tolerance was induced.³⁴ After removing piglets from a gestating MMP under general anesthesia, we injected human iPSCs or iPSC-derived cardiomyocytes into the thymus of the piglets. After rearing these piglets for 1 month, the challenge was repeated by grafting similar cells subcutaneously under the renal capsule and into the thymus. However, the transplanted human cells were clearly subject to immunorejection.

Actively acquired tolerance has been induced between different species, including between humans and sheep.³⁵ In this study, human mesenchymal stem cells were implanted into a sheep fetus, and the lamb was reared to maturity and then shown to be a human–sheep chimera. Fujiki et al. administered human cord blood to fetal

Fig. 2 Total thymectomy in the neonatal pig.

(A) Caesarean section in micro-mini pigs. (B) Total thymectomy under microscopic guidance. (C) Total view of the neonatal thymus. (D) The treated neonates were kept under specific-pathogen-free conditions.

pigs.³⁶ In all of these studies, pathological verification after growth of the fetus and genetic analysis by PCR, among other techniques, demonstrated that cells from the donor were in a chimeric state. These attempts to induce actively acquired tolerance have further attracted attention for their potential in postnatal acceptance of human cells.³⁷ Therefore, we plan to transplant human cells into fetal MMPs to induce actively acquired tolerance.

Concluding Remarks

Research on the production of human organs in the bodies of pigs has accelerated because of recent progress in human stem cell research and developmental engineering techniques. In pigs subjected to these techniques, immune tolerance to cells of human origin or a non-immunoreactive state has been induced, and such pigs may be useful as a preclinical system for verifying the safety and effect of cells intended for human regenerative medicine. Safety of human cells/tissue developed in pigs will need to be tested from the viewpoint of infectious disease and

the risk of malignancy, among others, before transplantation in patients can be considered.

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Fig. 3 Development of operational SCID Pigs.

(A) Schematic showing the procedure for developing an operational SCID pig. (B) Photograph of thymectomy in a mature mini-pig model. (C) Photograph of the resected spleen in a mature mini-pig model. Adapted from Itoh et al., *Nature Communications*, 2019 (DOI:10.1038/s41467-019-10107-1), **Fig. 1**. (Copyright 2019 Itoh et al. Used under the Attribution 4.0 International CC BY 4.0 License: <https://creativecommons.org/licenses/by/4.0/>).

were approved by the Ethics Committee of Keio University School of Medicine.

Conflicts of Interest

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